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(54) Title: USE OF NON-ANTICOAGULANT HEPARIN FOR TREATING ISCHEMIA/REPERFUSION INJURY

(57) Abstract

A method for treating or preventing ischemia/reperfusion in an animal host consisting of administering to the animal host, preferably either orally or intravenously, an effective amount of a non-anticoagulant heparin.

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USE OF NON-ANTICOAGULANT HEPARIN FOR TREATING ISCHEMIA/REPERFUSION INJURY

Field of the Invention

This invention is in the field of cardiovascular diseases. More specifically, it is directed to beneficial applications of essentially non-anticoagulant heparin oligomers for treating or preventing ischemia/reperfusion tissue injury in an animal.

Abbreviations

The following abbreviations are used for monosaccharides or for monosaccharide residues included in oligomers: D-glucuronic acid = GlcA; L-iduronic acid = IdoA; D-glucosamine = GlcNH2; N-acetyl-D-glucosamine = GlcNAc; D-glucosamine N-sulfate = GlcNS; 2,5-anhydromannose = AMan; 2,5-anhydromannitol = AManH.

In designating each saccharide residue, below the appropriate abbreviation, the location of the O-linked sulfate residues is indicated by "S" and the number of the position of sulfation where the sulfate residue is linked to oxygen on the sugar residue. In the designations for heparin structure, also, the positions involved in the alpha and beta anomeric linkages are as those conventionally found in heparin, α (glucosamine → uronic) and ß (uronic → glucosamine), and the D or L configurations as conventionally found pertains. The locations of the sulfates are shown below the abbreviation for the sugar to which they apply, thus, for example,

IdoA-GlcNS
2S 6S

refers to a dimer of L-iduronic acid and D-glucosamine N-sulfate-linked ß(1-4) with sulfates

connected respectively at the 2 and 6 positions of the sugar residues.

Background Art

Ischemia/Reperfusion Injury

Tissue injury caused by ischemia and reperfusion is a common surgical problem. The ischemia-reperfusion syndrome contributes significantly to increased morbidity, limb loss, and death. To date, the most effective therapy is fasciotomy. Other therapies include intravenous mannitol infusion which may reduce the generation of toxic oxygen free radicals that occur and damage tissue during reperfusion. McCord, J.M. New England Journal of Medicine (1985) 312:159-163. Other free radical scavengers have also been used including superoxide dismutase.

Additionally, several studies have shown that heparin may be beneficial and reduce muscle necrosis caused by the ischemia/reperfusion syndrome. Hobson, R.W. et al. Journal of Vasc. Surg. (1988) 7:585-591. However, heparin's effectiveness in treating ischemia-reperfusion is counter balanced by potential increases in bleeding associated with its administration. Wright, J.G. et al. Archives of Surgery (1988) 123:470-472.

Heparin

Heparin/heparan sulfate is a member of a class known as glycosaminoglycans (GAG). These materials are copolymers of alternating hexosamine and aldouronic acid residues which are found in sulfated forms and are synthesized as proteoglycans. In the compositions of interest herein, heparan sulfate and heparin, the hexosamine is mostly N-acetylated or N-sulfated glucosamine (GlcNAc and GlcNS), and the

aldouronic acid is mostly L-iduronic in heparin and mostly D-glucuronic acid in heparan sulfate. Heparan sulfate is commonly considered to have a higher proportion of glucuronic acid than heparin.

Problems of heterogeneity in preparations of heparan sulfate or heparin isolated from tissues make sharp distinctions difficult. Conventional heparin (used as an anticoagulant) has a molecular weight of 5-25 kd and is extracted as a mixture of various chain lengths by conventional procedures. These procedures involve autolysis and extraction of suitable tissues, such as beef or porcine lung, intestine, or liver, and removal of nonpolysaccharide components. The molecular weight of the chains in the extract is significantly lower than the 60-100 kd known to exist in the polysaccharide chains of the heparin proteoglycan synthesized in the tissue. The GAG moiety is synthesized bound to a peptide matrix at a serine residue through a tetrasaccharide linkage region of the sequence D-GlcA-D-Gal-D-Gal-D-Xyl → protein, which is then elongated at the D-GlcA residue with alternate additions of GlcNAc and GlcA.

Due to their obvious chemical similarity, isolated "heparin" may contain considerable amounts of what might otherwise be classified as heparan sulfate.

Non-anticoagulant Heparin

There is a body of art that describes the production of non-anticoagulant heparin. Most of the publications describe non-anticoagulant heparin produced from depolymerized heparin/heparan sulfate, and separation of products by size. In a generally used procedure, the heparin starting material is depolymerized in the presence of nitrous acid with or without pretreatment to remove acylation from any

GlcNAc residues present. Nitrous acid, under the appropriate conditions, cleaves at the linkage between a GlcNS or $GlcNH_2$ residue and the uronic acid residue through which it is linked through a glucosamine α(1-4) uronic acid linkage. If the heparin has been deacetylated, all of the glucosamine → uronic acid residues are susceptible and complete depolymerization results in disaccharides. If the heparin has not been deacetylated, the glucosamine → uronic acid residues wherein the glucosamine is acetylated are resistant, and both disaccharides and tetrasaccharides containing the resistant linkage result. In all cases, the glucosamine residue at the reducing terminus of the disaccharide or tetrasaccharide is converted to a 2,5-anhydromannose in the course of cleavage. This residue may further be reduced to the corresponding 2,5-anhydromannitol. These methods have been described by Bienkowski, M.J. and Conrad, H.E., J Biol Chem (1985) 260:356-365; Guo, Y., et al., Anal Biochem (1988) 168:54-62; and Guo, Y. and Conrad, H.E., Analyt Biochem (1989) 176:96-104. These methods are useful in analyzing the structure of heparin and in assessing the results of various treatments of the heparin chains. Further, there have been considerable attempts to use the products of degradation of heparin from both complete and partial digestion with nitrous acid as described in the foregoing papers, or from heparinase digestion or from periodate oxidation followed by ß-elimination. All of these processes generate low molecular weight heparins for therapeutic use.

An example of non-anticoagulant depolymerized low molecular weight heparin is described in U.S. Patent No. 4,990,502. It shows the treatment of heparin with periodate, followed by depolymerization with base, and reduction of the aldehydes generated

in the periodate treatment. The resulting material is said to contain a mixture of polymers containing 17-33 residues and containing a multiplicity of residues of the formula

IdoA-GlcAc
2S
or
IdoA-GlcNS
2S

wherein the glucosamine residue is sulfated at the 2 and/or 6 position in an arbitrary manner, and wherein some of the IdoA residues may be replaced by cleaved

IdoA
2S

or GlcA residues resulting from the periodate oxidation. These shortened polymeric chains are said to lack the binding site for ATIII but to be capable of inhibiting smooth muscle proliferation and to have physiological activities that include acceleration of tissue repair, prevention of atherogenous lesions, prevention of states of shock, and prevention of the development of metastasis.

Treatment of heparin/heparan sulfate with periodate has also been reported by others. Fransson, L.-A. and Lewis, W., FEBS Lett (1979) 97:119-123, describe a variety of conditions relating to the treatment of heparin/heparan sulfate with periodate and reduction by sodium borohydride or fragmentation in alkaline medium. Fransson concluded (erroneously as will be shown hereinbelow) that the glucuronic acid residues were preferentially oxidized as compared to iduronic acid residues, and that complete cleavage of all susceptible uronic acid residues, which is said to result in pronounced fragmentation of the molecule, resulted in the absence of anticoagulant activity.

Fransson, L.-A. et al., Carbohydrate Res (1980) 80:131-145, studied chemistry of various forms of treatment of heparin with periodate. In one study, the treatment with periodate is followed by ß-elimination in base to produce fragmentation. They further report treatment of heparin with periodate followed by partial acid hydrolysis which results in fragmentation of the chains and partial destruction of the functional groups.

Another example of an anticoagulant heparin is described by Casu, B. et al., Arzneim Forsch/Drug Res (1986) 36:637-642. They studied the effect of periodate oxidation on the anti-lipemic (lipoprotein lipase-releasing) activity of heparin. In this study, the heparin was oxidized with periodate and the products were reduced with borohydride. Although the authors stated that the product has the same molecular weight as the starting material, it is apparent from the figures presented in the paper that there is significant depolyermization.

Non-Anticoagulant Heparin and Treatment of Ischemia/Reperfusion Injury

As mentioned above, several authors have described the effects of heparin on ischemia/reperfusion injury. These studies, however, should not be confused with the effects of non-anticoagulant heparin (NAC heparin) on ischemia/reperfusion injury, which is the subject of the instant invention. To date, as far as we are aware, the use of non-anticoagulant heparin to treat ischemia/reperfusion injury has not been reported. Indeed, the scientific literature is ambiguous regarding the beneficial application of non-anticoagulant heparin for treating ischemia/reperfusion.

For example, Wright, J. et al., Arch. Surg. (1988): vol. 123; pages 470-472, studied the effect of heparin in an isolated gracilis muscle model, and demonstrated that heparin reduced the infarct size in heparinized dogs. They concluded that the mechanism of this protective effect may be due to heparin's anticoagulant...action."

Hobson, R. et al., Symposium: Fundamental Problems in Vascular Surgery I, 1988: vol. 7 no. 4; pages 585-590, also studied the effect of heparin in an isolated gracilis muscle model, and, similar to the work of Wright, J. et al., demonstrated that heparin reduced the infarct size in heparinized dogs. However, contrary to Wright's conclusion Hobson et al concluded that ... heparin's non-anticoagulant action might be more important in its protection of acutely ischemic skeletal muscle."

Ricci, M. et al., J. of Invest, Surg. 1990: vol. 3; pages 129-140, studied the effects of heparin on ischemia/reperfusion injury and concluded that ...the exact mechanism remains unknown."

A more recent study by Wright, J. et al., J. Cardiovasc. Surg. (Session 3: Ischemia Reperfusion) 1991: 3.7; page 11, extended their previous work on the beneficial effects of heparin for treating ischemia/reperfusion injury but concluded that Further investigations of the many properties of heparin are required to elucidate the mechanisms of its protective effect on ischemia/reperfusion injury in skeletal muscle."

The above studies do not elucidate if the effects of heparin are due to the anticoagulant or nonanticoagulant activities of heparin. Inherent in using anticoagulant heparin for treating ischemia/reperfusion are complications associated with bleeding. Thus, heparin or heparin fragments

that lack anticoagulant activity, but maintain properties that are beneficial for treating ischemia/reperfusion injury would have significant medical advantages.

Disclosure of the Invention

In one aspect, the invention is directed to a method of treating or preventing ischemia/reperfusion tissue injury in an animal host consisting of administering an effective amount of a non-anticoagulant heparin.

A second aspect of the invention consists of a method of treating or preventing ischemia/reperfusion tissue injury in an animal host by administering an effective amount of a non-anticoagulant heparin, preferably via intravenous or oral administration.

A third aspect of the invention is directed to a method of treating or preventing ischemia/reperfusion tissue injury in an animal host consisting of administering an effective amount of a substantially non-fragmented non-anticoagulant heparin.

A fourth aspect of the invention is directed to a method of treating or preventing ischemia/reperfusion tissue injury in an animal host consisting of administering an effective amount of a non-anticoagulant heparin which is produced from heparin by oxidation with periodate and subsequent reduction.

These and other aspects of the invention will be more fully understood upon a detailed consideration of the invention presented below.

Brief Description of the Figures

Figures 1A and 1B show the time course of periodate oxidation of heparin under two conditions: pH 3, 4°C and pH 6.5, 37°C.

Figures 2A-2D show the effect of periodate oxidation on various moieties in heparin at pH 3, 0°C.

Figure 3 shows the experimental design for perfusing non-anticoagulant heparin to ischemic rat limbs. Arrows below the time line indicate measurement of flow rates which were subsequently used to calculate the total vascular resistance of the hindlimb. Phenylephrine was added directly to the perfusate, and acetylcholine (0.001, 0.01, 0.1. 1.0 ug/min) and nitroprusside (0.5, 5.0, 50 ug/min) were delivered by constant infusion into the arterial cannula. With the exception of the 60 minute ischemic interval, non-ischemic and ischemia/reperfusion groups were treated identically.

Figure 4 compares the per cent change in total vascular resistance as a function of various concentrations of acetylcholine for post-ischemic or control animals treated with or without non-anticoagulant heparin or dextran sulfate.

Modes of Carrying Out the Invention

The invention relates to methods for treating ischemia/reperfusion injury in an animal host with a non-anticoagulant heparin. Preferably the non-anticoagulant heparin is substantially non-fragmented and prepared from commercially available heparin. More preferred the non-anticoagulant heparin is prepared by oxidization with periodate and reduction by sodium borohydride. Described herein are methods to prepare these compositions and the nature of the resulting composition.

It is important to note, and a skilled practitioner of this art will realize, that the methods of the instant invention can utilize a number of non-anticoagulant heparins. Thus, non-

anticoagulant heparins described by Fransson, L.-A. and Lewis, W., FEBS Lett (1979) 97:119-123, or in U.S. Patent No. 4,990,502, or by Casu, B. et al., Arzneim Forsch/Drug Res (1986) 36:637-642 may be used with the methods of the instant invention.

Throughout the specification reference is made to certain scientific publication, patents or patent applications. It is the intent of the applicants that these references be incorporated in their entirety into the application.

By "heparin/heparan sulfate" or "heparin" is meant a preparation obtained from tissues in a manner conventional for the preparation of heparin as an anticoagulant or otherwise synthesized and corresponding to that obtained from tissue. See Conrad, H.E., Heparin and Related Polysaccharides, Vol. 56, p. 18 of Annals of N.Y., Academy of Sc., June 7, 1989, incorporated herein by reference. This preparation may include residues of D-glucuronic acid (GlcA), as characteristic of heparan sulfate as well as iduronic acid (IdoA) as characteristic of heparin. However, both GlcA and IdoA are present in both, they are present in different proportional amounts. The (IdoA)/GlcA ratio rises as heparan sulfate becomes more heparin-like. As described in the Background section above, the conversion of D-glucuronic acid to L-iduronic acid is a result of epimerization at the 5 carbon of GlcA residues in a heparan-type intermediate. This sequence of steps involved in such epimerization and conversion is understood in the art. To the extent that full conversion has not been made, heparan sulfate characteristics remain in the preparation. Because the precise nature of the polymeric chains in the preparations of heparin is not generally determined, and varies from preparation to preparation, the term "heparin/heparan sulfate" or

"heparin" is intended to cover the range of mixtures encountered. Perhaps the main feature which distinguishes heparan sulfate from heparin is that the latter has anti-coagulant activity.

The "heparin/heparan sulfate" or "heparin" preparation can be obtained from a variety of mammalian tissues, including, if desired, human tissue. Generally, porcine or bovine sources are used, and vascularized tissues are preferred. A preferred source of heparin starting material is porcine intestinal mucosa, and preparations labeled "heparin" prepared from this tissue source are commercially available. In general, the heparin starting material is prepared from the selected tissue source by allowing the tissue to undergo autolysis and extracting the tissue with alkali, followed by coagulation of the protein, and then precipitation of the heparin-protein complex from the supernatant by acidification. The complex is recovered by reprecipitation with a polar nonaqueous solvent, such as ethanol or acetone or their mixtures, and the fats are removed by extraction with an organic solvent such as ethanol and proteins by treatment with a proteolytic enzyme, such as trypsin. Suitable procedures for the preparation of the heparin starting material are found, for example, in Charles, A.F., et al., Biochem J (1936) 30:1927-1933, and modifications of this basic procedure are also known, such as those disclosed by Coyne, E., in Chemistry and Biology of Heparin, Elsevier Publishers, North Holland, New York, Lunblad, R.L., et al., eds. (1981).

"NAC-heparin" refers to a mixture of substantially non-anticoagulant fragmented or non-fragmented heparin obtained by subjecting commercially available heparin to one or more

chemical treatments. Where stated in the specification, it refers to periodate oxidized heparin as described herein, which mixture substantially lacks anticoagulant activity and inhibits the proliferation of smooth muscle cells.

The invention method uses NAC-antiproliferative heparin to treat or prevent tissue injury associated with ischemia/reperfusion. The preferred NAC-antiproliferative heparin is described in co-owned U.S. Patent Application Serial No. 753,299, filed September 3, 1991. An additional preparation is described in co-owned U.S. Patent Application Serial No. 677,737, filed March 29, 1991.

In U.S. Patent Application Serial No. 753,299, the heparin starting material is treated with periodate under conditions wherein the diols on adjacent carbons contained in the glycosaminoglycan structure are oxidized to the corresponding aldehydes. Any glucuronic acid or iduronic acid which does not contain either or both a 2-sulfate or 3-sulfate would therefore be "susceptible" to oxidation and cleavage. However, as shown hereinbelow, the susceptible iduronic acid residues are cleaved much more rapidly than the susceptible glucuronic acid residues. Furthermore, susceptible glucuronic acid residues which are conjugated to the reducing terminus of a GlcNS residue are much less rapidly cleaved and oxidized than those susceptible glucuronic acid residues which are linked to the reducing terminus of a GlcNAc residue. Thus, the glucuronic acid residues which reside in the typical ATIII binding saccharide sequence are preferentially oxidized, and by suitable adjustment of the time course of oxidation, a large proportion of the ATIII binding activity can be destroyed without affecting in a substantial way, the antiproliferative activity.

Any free amino groups on glucosamine residues will also be oxidized; however, the majority of glucosamine residues in heparin are either sulfated or acetylated. These residues, therefore, are not affected by the periodate oxidation.

The periodate oxidation is followed by reduction of the resulting aldehydes to alcohols under conditions wherein fragmentation of the glycosaminoglycan polymer does not take place. The resultant nonanticoagulant (NAC) heparin derivative retains antiproliferative activity vis-à-vis smooth muscle cells.

In general, periodate oxidation is performed in 0.01-0.10 M sodium periodate buffered to a pH of 3-6, preferably with 0.05-0.2 M sodium acetate or sodium phosphate buffer. Reaction mixtures containing commercially-available heparin/heparan sulfate at 0.5-10% (wt./volume) are incubated with the periodate oxidation solution at 0-37°C in dark amber containers for time intervals generally greater than 3 hrs. While this temperature range is workable, lower temperatures are greatly preferred, especially in the range of 0°-5°C, especially 0°-1°C. Shorter reaction times are needed for temperatures and pH values in the higher range, longer reaction times may be used for lower pH and temperatures In order to properly control the oxidation to provide the required diminution of anticoagulant activity while retaining antiproliferative properties, low pH and low temperatures are preferred so that the course of the reaction may be more accurately controlled. Excess periodate is then destroyed by addition of 100-500 mM ethylene glycol, and the reaction mixture is dialyzed against water.

Reduction is immediately effected with approximately 0.2 M of a suitable aldehyde-reducing agent, such as sodium borohydride at pH 8.5-9.5. Sodium bicarbonate buffer at approximately 0.2 M can appropriately be used to maintain this pH. It is important that the pH not be higher so that ß-elimination is prevented. The concentration of the oxidized heparin in the reduction mixture is 1-20% (w/v). Excess borohydride is then destroyed by addition of concentrated HCl to approximately pH 4. The pH is then readjusted to neutrality with 2 M sodium carbonate and the product is desalted and dried.

The resulting composition contains modified but unfragmented heparin/heparan sulfate of molecular weight in the range of 5-25 kd with an average chain length of 50-100 saccharide units. The composition is a mixture of oxidation products corresponding to the original mixture of glycosaminoglycans in the heparin preparation, but is free of other biological contaminants. The composition is useful therapeutically under circumstances where antiproliferative activity is desirable. In a typical preparation, the anticoagulant activity of the original heparin/heparan sulfate preparation is reduced to less than 40 u/mg, preferably less than 5 U/mg, as opposed to 170 U/mg in the original preparation. The inhibition of smooth muscle cells by the preparation is the same as or greater than that of the original heparin on a weight basis.

In U.S. Patent Application Serial No. 677,737, filed March 29, 1991, the heparin starting material is subjected to two distinct chemical reactions to convert it to a non-anticoagulant heparin. The method consists of first N-deacetylating N-acetylhexosamine, followed by a second reaction which consists of

oxidizing deacetylated heparin, preferably with periodate.

Generally, the reaction consist of dissolving heparin (Ming Han heparin, 170Umg) in distilled water to give 540 ml of a solution containing 80 g heparin. Eighteen grams of hydrazine sulfate are dissolved in 1260 ml of anhydrous hydrazine in a 3 liter beaker and the heparin solution added to the beaker with stirring. The reaction is allowed to proceed for 6 hours at about 98°C, and the deacetylated product is isolated. The deacetylated heparin is next oxidized in solution containing 0.5-10% deacetylated heparin (w/v) with 0.01-0.10 M periodate at pH3-6 at 0°-37° for a time sufficient to affect complete oxidation of heparin by periodate. Finally, the aldehyde groups produced are reduced to alcohols by treatment with sodium borohydride at about 0.1-0.3 M, pH 8.9.

A technical point is worth noting regarding the N-deacetylation reaction. Hydrazine is used to deacetylate heparin. Because hydrazinolysis has the unwanted side effect of reacting with carboxyl groups on heparin which causes fragmentation of heparin, to avoid fragmentation the reaction mixture is incubated with IO_3 (not to be confused with periodate, IO_4).

Treatment or Prevention of Ischemia-Reperfusion Injury

A number of animal models systems may be utilized to show the efficacy of NAC heparin for treating or preventing ischemia-reperfusion tissue injury. For example, a canine skeletal muscle ischemia/reperfusion model is described by Hobson et al. in Journal of Vasc. Surg. (1988) 7:585-590. A rat hindlimb ischemia/reperfusion model is described by Wright et al. Journal Cardiovasc. Surg. (1991) 32 (for supplement):11. An additional rat model system

is described by Sternberg and Edelman, Journal Vasc. Surg. (1992) 16:30-39.

The efficacy of NAC heparin for treating or preventing ischemia-reperfusion tissue injury in the instant invention was demonstrated using an isolated rat hindlimb model. This model system, as well as the others mentioned above, are correlative and predicative of the efficacy of NAC heparins in animals including humans. Briefly, the procedure consists of perfusing isolated rat hindlimbs with an appropriate physiological solution and causing ischemia by halting perfusate flow. A contralateral limb serves as a control which is subjected to constant perfusate flow. See Sternberg et al. Journal of Vasc. Surg. (1992) 16:30-39.

The NAC-heparin treated limb and the control limb are treated identically except the former is subjected to sixty (60) minutes of ischemia, and is perfused with pre- and post-ischemic perfusate containing NAC-heparin. To determine the beneficial effects of NAC heparins on ischemia/reperfusion injury, its effects on both endothelial cell-dependent and independent vasoreactivity is determined by measuring the change in vascular resistance in response to increasing amounts of acetylcholine and nitroprusside, respectively, infused into the artery.

Intact vasculature of an organ must be preconstricted to facilitate measurement of vasodilator responses to agonists. Amezcua, J. et al., Br. J. Pharmacol(1989): vol.97; pages 1119-1124. For the instant measurements, the base line was established by measuring native venous flow rates of non-ischemic or ischemic limbs by perfusing the limbs with Kreb's buffer containing phenylephrine for 10 minutes to pre-constrict the vasculature. This pre-

constricted flow rate was then recorded which was used as the base line for subsequent measurements.

Total vascular resistance may be calculated by dividing the perfusion pressure by the flow rate and expressed as mm Hg/ml/min/g dry limb weight. The results can be expressed as the percent reduction in endothelial-dependent vasal dilation to acetylcholine when compared to the non-ischemic controls.

More specifically, rat hindlimbs in both the experimental and control groups are perfused with Kreb's buffer for twenty (20) minutes. NAC-heparin is included in the perfusate experimental group which is subsequently subjected to sixty (60) minutes of ischemia. Next, reperfusion is conducted for ten (10) minutes with NAC heparin. Endothelial cell function is assessed in both the experimental and control groups as a function of total vascular resistance in response to various concentrations of acetylcholine. Endothelial cell independent vasoreactivity is assessed with nitroprusside.

Labeled Forms of the Invention

Non-Anticoagulant Compositions

The non-anticoagulant compositions of the invention can be provided with fluorescent, radioisotope, or enzyme labels as desired. Conventional techniques for coupling of label to carbohydrates or related moieties can be used. Such techniques are well established in the art. See, for example, U.S. Patent No. 4,613,665. The labeled mixtures of the invention may be used to identify sites of ischemia as well as in competitive immunoassays, and as a means to trace the pharmacokinetics of the compositions in vivo. Suitable radioisotope labels for this purpose include hydrogen^{3}, iodine^{131}, indium^{111}, technetium^{99}, and

phosphorus^{32}. Suitable enzymic labels include alkaline phosphatase, glucose-6-phosphate-dehydrogenase, and horseradish peroxidase. Particularly preferred fluorescent labels include fluorescein and dansyl. A wide variety of labels of all three types is known in the art.

Administration and Use

The non-anticoagulant heparin compositions of the invention are useful in therapeutic applications for treating or preventing tissue injury caused by ischemia/reperfusion.

Administration is by typical routes appropriate for glycosaminoglycan compositions, and generally includes systemic administration, such as by injection. Particularly preferred is intravenous injection, as continuous injection over long time periods can be easily continued. Also preferred are introduction into the vascular system through intraluminal administration or by adventitial administration using osmotic pumps or implants. Typical implants contain biodegradable materials such as collagen, polylactate, polylactate/polyglycoside mixtures, and the like. These may be formulated as patches or beads. Typical dosage ranges are in the range of 0.1-10 mg/kg/hr on a constant basis over a period of 5-30, preferably 7-14, days. Particularly preferred dosage is about 0.3 mg/kg/hr, or, for a 70 kg adult, 21 mg/hr or about 500 mg/day.

Other modes of administration are less preferred but may be more convenient. Injection subcutaneously at a lower dose or administered orally at a slightly higher dose than intravenous injection, or by transmembrane or transdermal or other topical administration for localized injury may also be effective. Localized administration through a

continuous release device, such as a supporting matrix, perhaps included in a vascular graft material, is particularly useful where the location of the trauma is accessible.

Formulations suitable for the foregoing modes of administration are known in the art, and a suitable compendium of formulations is found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, latest edition.

The compositions of the invention may also be labeled using typical methods such as radiolabeling, fluorescent labeling, chromophores or enzymes, and used in a competitive assay for the amount of antiproliferative component in a biological sample. Suitable protocols for competitive assays of analytes in biological samples are well known in the art, and generally involve treatment of the sample, in admixture with the labeled competitor, with a specific binding partner which is reactive with the analyte such as, typically, an immunoglobulin or fragment thereof. The antibodies prepared according to the invention are useful for this purpose. The binding of analyte and competitor to the antibody can be measured by removing the bound complex and assaying either the complex or the supernatant for the label. The separation can be made more facile by preliminary conjugation of the specific binding partner to a solid support. Such techniques are well known in the art, and the protocols available for such competitive assays are too numerous and too well known to be set forth in detail here.

The antibodies of the invention are useful in immunoassays, not only of the type described above involving competition between labeled composition and the analyte antiproliferation factor in the sample, but also for direct immunoassay for the factor.

Alternate protocols involving direct assays are also of wide variety and well known. Typically, the analyte bound to antibody is detected by means of an additional reactive partner which bears a label or other means of detection. Thus, in typical sandwich assays, for example, the binding of the antibodies of the invention to analyte can be detected by further reaction with a labeled preparation of these same antibodies or by labeled antibody immunoreactive with this preparation by virtue of species differences.

The antibodies of the invention can also be formulated into pharmaceutical compositions and used to stimulate the growth of smooth muscle cells in subjects for which this result is desirable.

Examples

The following examples are intended to illustrate but not to limit the invention.

Example 1

Preparation of Nonanticoagulant (NAC) Heparin Mixtures

A. 20 g of porcine mucosa heparin (Ming Han heparin, 900201, 170 u/mg) was dissolved in 450 ml of distilled water and 50 ml of 1 M sodium acetate buffer, pH 5.2, was added. The solution was transferred to a 1 l amber bottle and chilled to 0°C in an ice bath. After the temperature had equilibrated 500 ml of prechilled 0.2 M sodium periodate was added with moderate stirring.

After 15 hr at 0°C, the mixture was warmed to room temperature and 16 ml of ethylene glycol was added. The mixture was incubated for 1 hr at room temperature to destroy excess periodate.

The solution of oxidized heparin was filtered through Whatman #1 paper to remove a small amount of

flocculent precipitate and the filtrate was dialyzed against four changes of distilled water (vol ratio 1:10) using a 3.5 kDa cutoff membrane. The volume was then reduced to 400 ml by ultrafiltration using a Pharmacia Tangential Flow Apparatus fitted with a Nova 1 kDa molecular weight cut-off membrane.

The concentrated solution was chilled to 0°C in an ice-water bath and 8.3 g of $NaHCO_3$ was added. A solution containing 3.78 g $NaBH_4$ in 100 ml of 0.05 M Na_2CO_3 pre-chilled to 0°C was added to the reaction mixture and the reduction was allowed to proceed at 0°C with moderate stirring. The pH of the reaction mixture was 8.5 at the beginning of the reaction and rose to 9.5 as the reaction proceeded. After 2 hr the pH was adjusted to 4.0 by addition of 6 N HCl and the mixture was allowed to stand for 30 min at room temperature to destroy the excess $NaBH_4$. Finally, the pH was adjusted to 7.0.

The solution containing the final product was dialyzed as described above and then lyophilized to dryness. The overall yield was 90% of the starting weight of heparin.

For further purification the product was dissolved in distilled water to give a 5% solution (wt/vol) and reprecipitated with 3 volumes of 99% ethanol. The precipitate was washed three times with 99% ethanol and the remaining ethanol was removed by placing the powder in the lyophilizer for 1 hr.

B. In a procedure using more dilute solutions, porcine heparin (Ming Han Batch HM900201) was periodate oxidized at a concentration of 0.8% of heparin in a reagent solution of 20 mM $NaIO_4$, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, and 0.2 M NaCl (pH 6.5). The reaction mixture was prepared by addition of 1500 ml of 1.6% heparin (25 grams) in deionized water to 1500 ml of 40 mM $NaIO_4$, 40 mM NaH_2PO_4-40 mM Na_2HPO_4, 0.4 M

NaCl with moderate stirring at room temperature. The mixture was transferred to three 1 liter brown bottles immediately after the mixing. The reaction was run at 37°C for 24 hrs in an incubator. After the reaction, excess periodate was consumed by adding 16 ml of ethylene glycol (to give a final concentration of 100 mM) to the reaction mixture and incubating at 37°C for 1 hr.

The reaction mixture was then concentrated to 475 ml by ultrafiltration using a Pharmacia Tangential Flow Apparatus with a 1000 Dacut-off membrane. The pH of concentrated mixture was adjusted to 8.5 with 2 M Na_2CO_3 and the oxidized heparin was then reduced by addition of 17 grams of $NaBH_4$ powder to the solution (to give a final concentration of 0.5 M $NaBH_4$). The reduction was run at 40°C for 1 hr. After the reduction, the reaction was stopped by adjusting the pH to 3 with concentrated HCl, and the mixture was allowed to stand for 30 min at room temperature. The pH of the final solution was then adjusted to 7 by addition of 50% of NaOH.

To the final solution (600 ml), 3 volumes of absolute ethanol were added with moderate stirring. The precipitant was collected by centrifugation at 4000 g for 20 min. The pellets were washed with absolute ethanol three times. The pellets were then lyophilized to dryness (the yield was 20 grams).

The pellets were dissolved in deionized water and dialyzed against water for 48 hrs in a 1000 Da cut-off dialysis tube with three changes of water. The dialyzed material was lyophilized to dryness to generate the nonanticoagulant heparin (7 grams) as the retentate. The solutions outside of the dialysis tube were combined, concentrated, dialyzed and

lyophilized to generate lyophilized dialysate (4 grams).

C. Preparation of nonanticoagulant heparin in absence of 0.2 M NaCl was carried out exactly as above, except that 0.2 M NaCl was absent during the periodate oxidation. This reaction gave 10 grams of retentate and 3 grams of dialyzate.

Example 2

Properties of the NAC-antiproliferative Compositions

The NAC-antiproliferative heparin prepared according to paragraph A of Example 1 shows less than 5 u/mg anticoagulant activity compared to 170 u/mg for the starting material.

The NAC-antiproliferative heparin preparation in paragraphs B and C were tested using intravenous delivery in the assay described hereinabove using 29 male Sprague Dawley FBR albino rats weighing approximately 350 grams. The animals in groups 1 and 2 received the NAC-antiproliferative heparin prepared as in paragraphs B and C, respectively, at the rate of 0.3 mg/kg/hr for 14 days in lactated Ringer's solution. Group 3 received lactated Ringer's solution alone. When percent occlusion was measured, it was found that for the 10 animals in the control group (3) the lumen of the carotid artery was occluded to the extent of 21% of its cross-sectional area (21% occlusion); for the nine animals in the group receiving the NAC-antiproliferative heparin prepared as set forth in paragraph C, 2.5% occlusion occurred; for the 10 rats in the group receiving the NAC-antiproliferative heparin prepared as in paragraph B of Example 1, 5.6% occlusion occurred.

Thus, the NAC-antiproliferative heparin was as effective in preventing myointimal hyperplasia as heparin. It was also observed that no visible

bleeding occurred post surgery in any of the rats, and the animals took less time to recover and looked healthier than those previously treated with whole heparin.

Additional studies on the NAC-antiproliferative preparation as set forth in paragraphs B and C of paragraphs 1 showed almost exclusively the presence of long oligomers with only a few shorter chains. Earlier preparations which resulted in significant depolymerization gave less satisfactory results.

The NAC-antiproliferative heparins prepared in Example 1 were also analyzed for disaccharide composition by complete hydrolysis in the presence of nitrous acid, as described in Guo, Y., and Conrad, H.E., Anal Biochem (1989) 176:96-104. Hydrolysis with nitrous acid cleaves at N-sulfated glucosamine residues (but not at N-acylated glucosamine residue) and converts the reducing terminus to 2,5-anhydromannose. Subsequent reduction of this residue to 2,5-anhydromannitol is used to stabilize the cleavage products in this assay. The various hydrolysis products are quantitated relative to

IdoA-AManH
2S 6S

which is known not to be destroyed in periodate oxidation and is set at 100.

A comparison of the composition of the NAC-antiproliferative composition with that of heparin is shown in Table 1. "ND" represents not detectable. As shown in Table 1, disaccharide segments susceptible to periodate oxidation (i.e., those containing unsulfated IdoA or GlcA) are completely destroyed. Those units expected not to be susceptible to periodate oxidation (those containing 2S IdoA or 2S GlcA) are retained at approximately the

same ratio to the resistant standard as their occurrence in heparin.

Table 1

Disaccharide	Heparin	NAC-antiproliferative
IdoA-AManH or GlcA-AManH	10.0	ND
IdoA-AManH 2S	18.0	22.8
GlcA-AManH 6S	18.0	ND
GlcA-AManH 2S	2.6	2.6
IdoA-AManH 6S	12.9	ND
GlcA-AManH 3S,6S	7.4	ND
IdoA-AManH 2S 6S	100	100

Example 3

Controlled Periodate Oxidation of Heparin

Hog mucosa heparin (anticoagulant activity = 170 USP units/mg) was oxidized with $NaIO_4$ at 4°C in 50 mM Na citrate buffer (pH 3.0) or at 37°C in 50 mM Na phosphate (pH 6.5), essentially as described by Fransson et al. Carbohyd Res (1978) 62:235-244; and Fransson et al., FEBS Lett (1979) 97:119-123. Aliquots were removed from the reaction mixtures at intervals and treated with ethylene glycol to destroy unreacted $NaIO_4$. The samples were dialyzed vs water, dried by lyophilization, and reduced with $NaBH_4$.

The disaccharide and tetrasaccharide compositions obtainable by degradation of the original heparin and of the oxidized samples were measured to follow the destruction of the uronic acid residues of the heparin during the oxidization. Each sample was treated with nitrous acid at pH 1.5 and the resulting di- and trisaccharides were quantified using the reversed phase ion pairing HPLC method described previously (Guo, Y. and Conrad, H.E. Analyt Biochem (1989) 176:96-104). Anticoagulant activity

was determined by APTT and anti-Xa assays. These assays were kindly performed by Dr. Betty Yan, Lilly Research Labs, Indianapolis, IN, USA.

The trisulfated disaccharide that is critical for the anticoagulant activity is

GlcA-GlcNS.
3S,6S

A tetrasaccharide, t14, that contains this disaccharide is

IdoA-GlcNAc-GlcA-GlcNS
6S 3S,6S

The trisulfated disaccharide, when released from the N-deacetylated heparin by treatment with nitrous acid, yields

GlcA-AMan
3S,6S

abbreviated here as GMS_2. Direct nitrous acid cleavage of this segment of heparin yields the tetrasaccharide, t14, and not free GMS_2. On the other hand, when the GMS_2 in heparin is situated in a position with a GlcNS residue linked to the C4 position of its GlcA, it will be released by nitrous acid without prior N-deacetylation as the free disaccharide.

Other possible degradation products and their abbreviations are:

ISMS: IdoA-AMan;
2S 6S

ISM: IdoA-AMan;
2S

IMS: IdoA-AMan;
6S

and

GMS: GlcA-AMan
6S.

Figure 1 shows a comparison of the rates of disappearance of the major disaccharide units of heparin at pH's 3.0 (4°C) (Figure 1a) and 6.5 (37°C) (Figure 1b). For these measurements, the samples at various time intervals were N-deacetylated and then cleaved with nitrous acid at both pH 1.5 and pH 4.0 to give total disaccharide release. Since all of the GlcN-type residues in heparin are resistant to $NaIO_4$ oxidation, the disappearance of each susceptible disaccharide is due to the oxidation of its uronic acid residue. Only those uronic acid residues that lack a SO_4 substituent at both C2 and C3are susceptible to IO_4^-.

The results in Figure 1 show (a) that the overall oxidation of susceptible uronic acids proceeds more rapidly at pH 6.5 and 37°C than at pH 3.0 and 4°C, and (b) that under both oxidation conditions the unsulfated IdoA residues are oxidized much more rapidly than the unsulfated GlcA residues. A study of heparin oxidation at pH 5 and 4°C (not shown) gave rates similar to those observed at pH 6.5. Since the ratios of the rates of IdoA and GlcA oxidation were similar at both pH's 3.0 and 6.5, the pH 3 conditions were chosen for further examination of the oxidation of heparin, since, under the latter conditions, the progression of the reaction could be observed over a more extended time interval, allowing better control of the reaction.

The slow and incomplete oxidation of GMS_2 at pH 3 suggests that the anticoagulant activity of heparin should also be lost slowly and incompletely, as reported previously (Fransson, L.-A. et al. Carbohyd Res (1980) 80:131-145). To explore this further, the

rates of loss of anticoagulant activity and GMS_2 were compared, as shown in Figure 2.

The results shown in Figure 2a (total disaccharides) are the same as those shown in Figure 1a. The GMS_2 and t14 released by pH 1.5 nitrous acid treatment without prior N-deacetylation are shown in Figure 2b. The GMS_2 disaccharide formed under the latter conditions represents "GlcNS-linked GMS_2"; i.e., this GMS_2 is formed only when there is a GlcNS residue linked to the GlcA. Oxidation associated with the acetylated form (t14) occurs much more rapidly. Subtraction of the GlcNS-linked GMS_2 of Figure 2b from the total GMS_2 of Figure 2a yields the GlcNAc-linked GMS_2, shown in Figure 2c, all or most of which is derived from the antithrombin III-binding pentasaccharide. This is confirmed in Figure 2d which shows loss of anticoagulant activity as measured by APTT or anti-Xa.

The results in Figures 2a-2d show that the GlcNAC-linked GMS_2 and the GlcNS-linked GMS_2 are oxidized at markedly different rates, and that the rate of loss of anticoagulant activity parallels the rates of disappearance of only the GlcNAc-linked GMS_2 and the t14 tetrasaccharide (which contains the GlcNAc-linked GMS_2), and not the GlcNS-linked GMS_2.

Example 4

Effects of NAC Heparin on Ischemia/Reperfusion Injury

Generally the procedures used to show the efficacy of NAC heparins for treating ischemia/reperfusion injury are described by Sternberg and Edelman in Journal Vasc. Surg. (1992) 16:30-39. The experimental design that was used is shown in Figure 3.

Briefly, a gravity fed perfusion apparatus that delivered a constant perfusion pressure was employed. The perfusate chambers and all tubing that delivered perfusate were kept in a waterjacket to maintain a constant temperature of 37°C. Ischemia was established by arresting perfusate flow to the limb.

Male Sprague-Dawley rats weighing 350-400 grams were used. Rats were anesthetized with pentobarbital 50 mgs/kg administered intraperitoneally. Skinned hindlimbs that were kept moist were utilized, and the femoral artery and vein were isolated immediately distal to the inguinal ligament. The artery was cannulated and flushed with albumin enriched Kreb's buffer. Next, the vein was also cannulated, and the limb amputated.

Venous flow from the hindlimb was measured by timed collection over a 30 second interval, normalized to ml/mIn, and expressed per gram dry limb weight (ml/min/g dry limb weight). Venous effluent was not recirculated. It is to be noted that because this ischemia/reperfusion injury model has a constant perfusion pressure, the total vascular resistance of the hindlimb can be calculated directly from the flow rate.

All limbs were initially perfused for a twenty (20) minute stabilization period. After measurement of the native venous flow, non-ischemic limbs were perfused with Krebs buffer plus phenylephrine for ten (10) minutes to preconstrict the vasculature. The preconstricted flow rate was then recorded and used as the baseline for subsequent measurements. Endothelial cell-dependent vasoreactivity was examined by measuring the change in flow in response to increasing log amounts of acetylcholine infused into the arterial perfusate line. Flow measurements were taken 2 minutes after the initiation of each

infusion. Endothelial cell-independent vasoreactivity was similarly assessed with nitroprusside after flow returned to its preconstricted baseline, about 2-3 minutes. Preliminary studies established that the order of injection does not affect limb vasoreactivity.

Hindlimbs that were subjected to ischemia/reperfusion were also subjected to an equivalent period of 20 minutes stabilization perfusion followed by sixty (60) minutes of total ischemia at 37°C. The limbs were reperfused for 10 minutes with buffer containing phenylephrine. Thereafter, vasoreactivity to acetylcholine and nitroprusside were tested as performed in the non-ischemic limbs. Thus, both the non-ischemic and ischemia/reperfusion hindlimbs were subjected to identical amounts of perfusion prior to administration of the agonists. In the NAC-heparin treated group, the drug was added to the pre-and post-ischemic perfusate.

At the termination of all studies, hindlimbs were weighed and placed in a drying oven at 50°C for 48 hours to determine dry weight and water content.

Acetylcholine was used at four concentrations: 0.001, 0.01, 0.1, and 1.0 μgms/min., and nitroprusside was used at three concentrations: 0.5, 5.0, and 50 μgms/min. NAC heparin was used at a concentration of 4 μgms/ml. Acetylcholine and nitroprusside were suspended in normal saline and delivered into the arterial perfusate line with an infusion pump. Flow rates became steady state after 10 minutes of perfusion with phenylephrine and 1-1.5 minutes with acetylcholine and nitroprusside.

Figure 4 shows the results. Post-ischemic limbs (n=12) had a 46.2% reduction in endothelial-dependent vasodilation to acetylcholine when compared to non-

ischemic controls (n=7, $p<0.05$). Perfusion with NAC-heparin (n=6) resulted in only a 12.3% reduction in endothelial-dependent vasodilation. This number is significantly less than the untreated post-ischemic group ($p<0.05$), and not statistically different from the non-ischemic control group.

In order to show that the effect of NAC-heparin is associated with structural specificity of the drug, a control compound, dextran sulfate, was used at a concentration of 4 ugms/ml. Dextran sulfate is a randomly sulfated polymer of comparable size to NAC-heparin. It was tested in an identical fashion as NAC-heparin. Figure 4 shows, most importantly, that dextran sulfate (n=7) exhibited a 34.3% reduction in endothelial-dependent vasodilation to acetylcholine when compared to non-ischemic controls. This finding strongly supports the conclusion that the unique structural properties of NAC-heparin account for its considerable beneficial effects for treating ischemia/reperfusion injury.

Endothelial cell-independent vasodilation to nitroprusside was not different in any group study, confirming that there was no direct damage to the vascular smooth muscle. Thus, it is important to note that the post-ischemic impairment of vasodilation and its protection by NAC heparin were specific to the endothelium.

Total vascular resistance (TVR) was calculated by dividing the perfusion pressure (70 mm Hg) by the flow rate and expressed as mmHg/ml/min/g dry limb weight. See, Sexton, W.L., et al. Journal of Applied Physiol. (1990) 68:387-392. The effect of NAC on TVR was expressed as a percent change from the phenylephrine-preconstricted base line. See, Mugge, A., et al. American Journal of Physio. (1991) 260:H242-H247.

Data was analyzed using a 1-way analysis of variance with Tukey's studentized range test for comparison of variance between any two groups. Groups were considered significantly if $p<0.05$. All data are presented as mean ± S.E.

Claims

1. A method for treating or preventing ischemia/reperfusion in an animal host in need of said treatment comprising administering to said animal host an effective amount of a non-anticoagulant heparin.

2. A method as described in claim 1 wherein said non-anticoagulant heparin is a substantially unfragmented derivative of heparin.

3. A method as described in claim 2 wherein said non-anticoagulant heparin is a substantially unfragmented derivative of heparin that has less than 3% of the anticoagulant activity of heparin.

4. A method as described in claim 3 wherein said non-anticoagulant heparin has a disaccharide composition comprising substantially no IdoA-AManH, GlcA-AManH, GlcA-AManH 6S, IdoA-AManH 6S, or GlcA-AManH 3S, 6S.

5. A method as described in claim 4 wherein administering said non-anticoagulant heparin is orally or intravenously.

6. A method for imaging tissue in an animal that has been subject to ischemia/reperfusion injury, comprising the steps:

administering to said animal a non-anticoagulant heparin; and

detecting the presence of said non-anticoagulant heparin.

7. A method as described in claim 6 wherein the presence of said non-anticoagulant heparin is

detected comprising labelling said heparin, and detecting said labelled heparin.

8. A method as described in claim 6 wherein the presence of said non-anticoagulant heparin is detected using a labelled antibody that binds to said non-anticoagulant heparin.

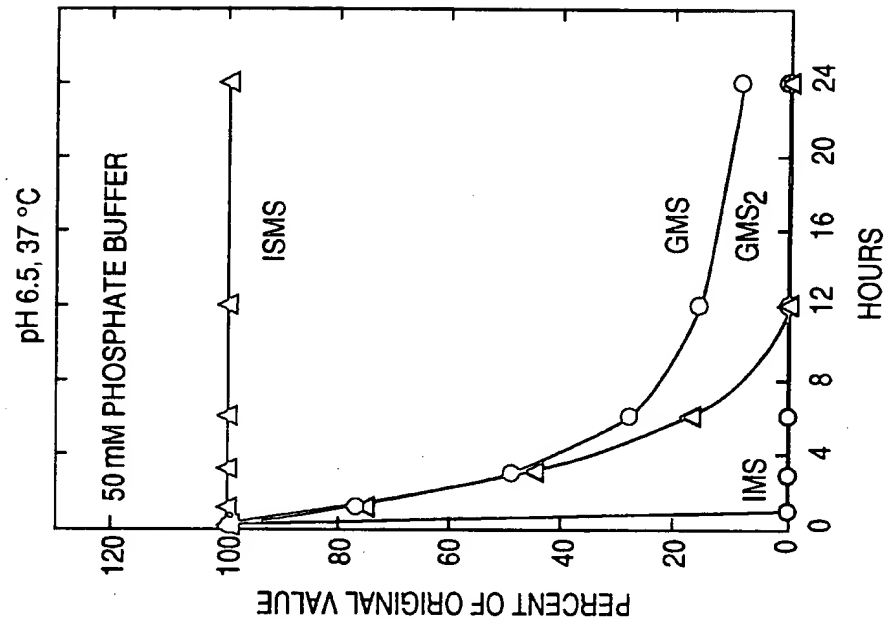
pH 6.5, 37 °C
50 mM PHOSPHATE BUFFER
ISMS
GMS
GMS2
IMS
PERCENT OF ORIGINAL VALUE
HOURS
0
20
40
60
80
100
120
0
4
8
12
16
20
24

FIG. 1B

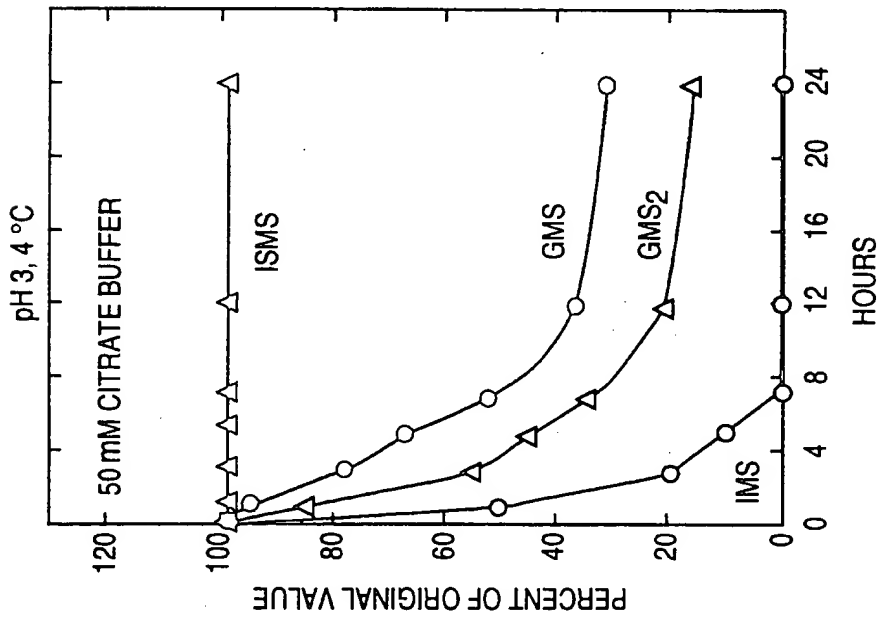
pH 3, 4 °C
50 mM CITRATE BUFFER
ISMS
GMS
GMS2
IMS
PERCENT OF ORIGINAL VALUE
HOURS
0
20
40
60
80
100
120
0
4
8
12
16
20
24

FIG. 1A

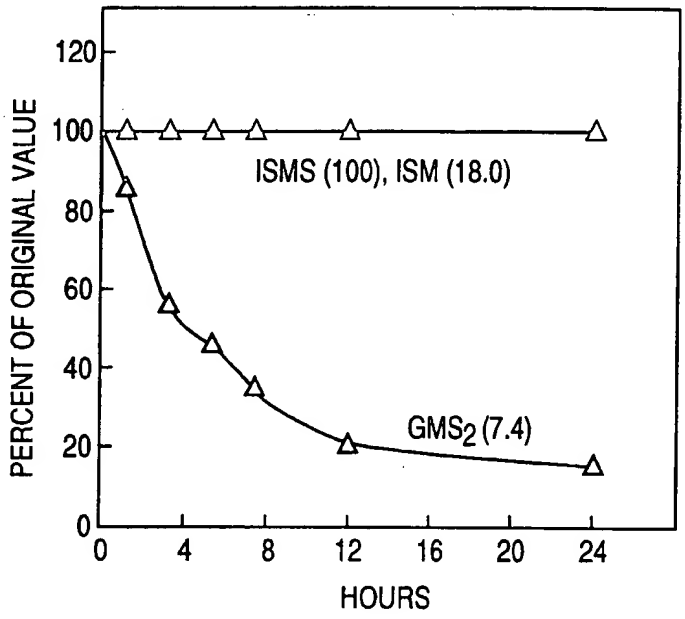
PERCENT OF ORIGINAL VALUE
120
100
80
60
40
20
0
0
4
8
12
16
20
24
HOURS
ISMS (100), ISM (18.0)
GMS2 (7.4)

FIG. 2A

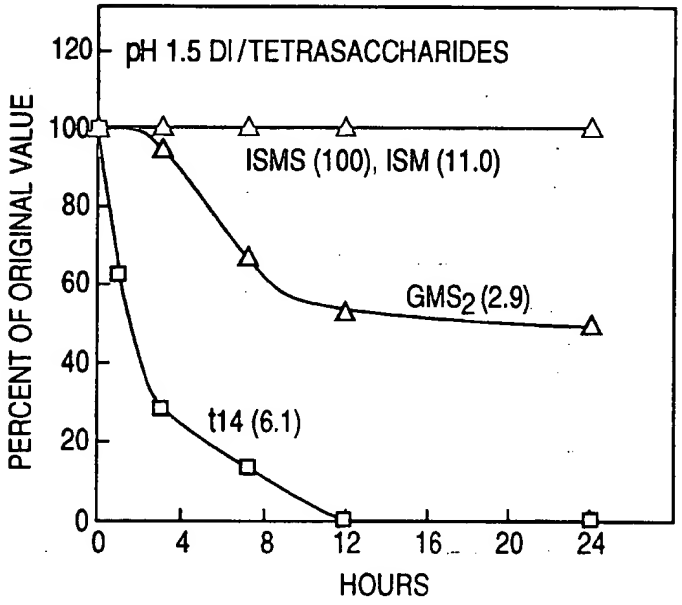
pH 1.5 DI/TETRASACCHARIDES
PERCENT OF ORIGINAL VALUE
120
100
80
60
40
20
0
0
4
8
12
16
20
24
HOURS
ISMS (100), ISM (11.0)
GMS2 (2.9)
t14 (6.1)

FIG. 2B

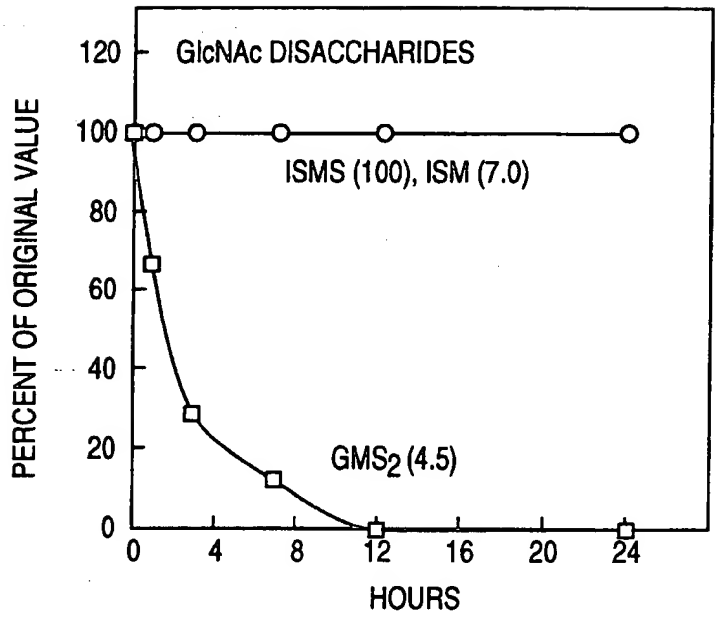
GlcNAc DISACCHARIDES
ISMS (100), ISM (7.0)
GMS2 (4.5)
PERCENT OF ORIGINAL VALUE
HOURS
120
100
80
60
40
20
0
0
4
8
12
16
20
24

FIG. 2C

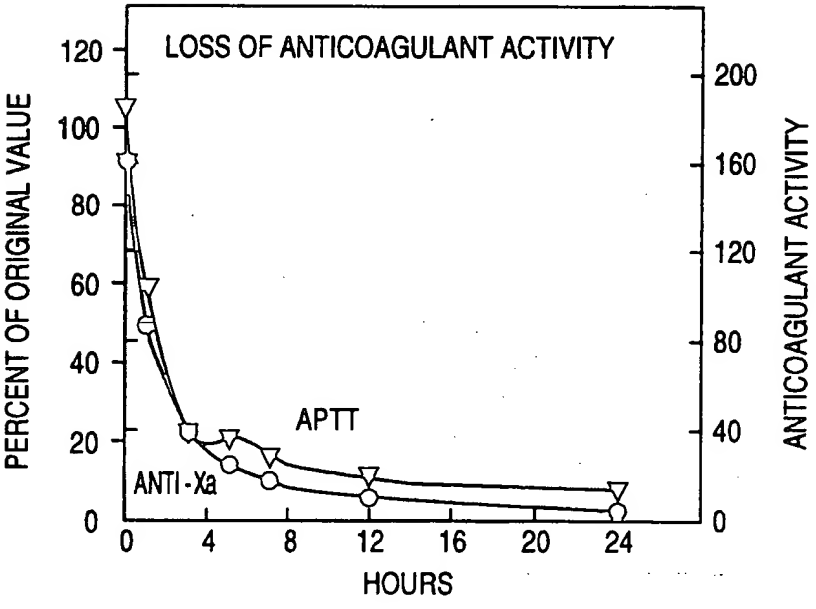
LOSS OF ANTICOAGULANT ACTIVITY
APTT
ANTI-Xa
PERCENT OF ORIGINAL VALUE
ANTICOAGULANT ACTIVITY
HOURS
120
100
80
60
40
20
0
200
160
120
80
40
0
0
4
8
12
16
20
24

FIG. 2D

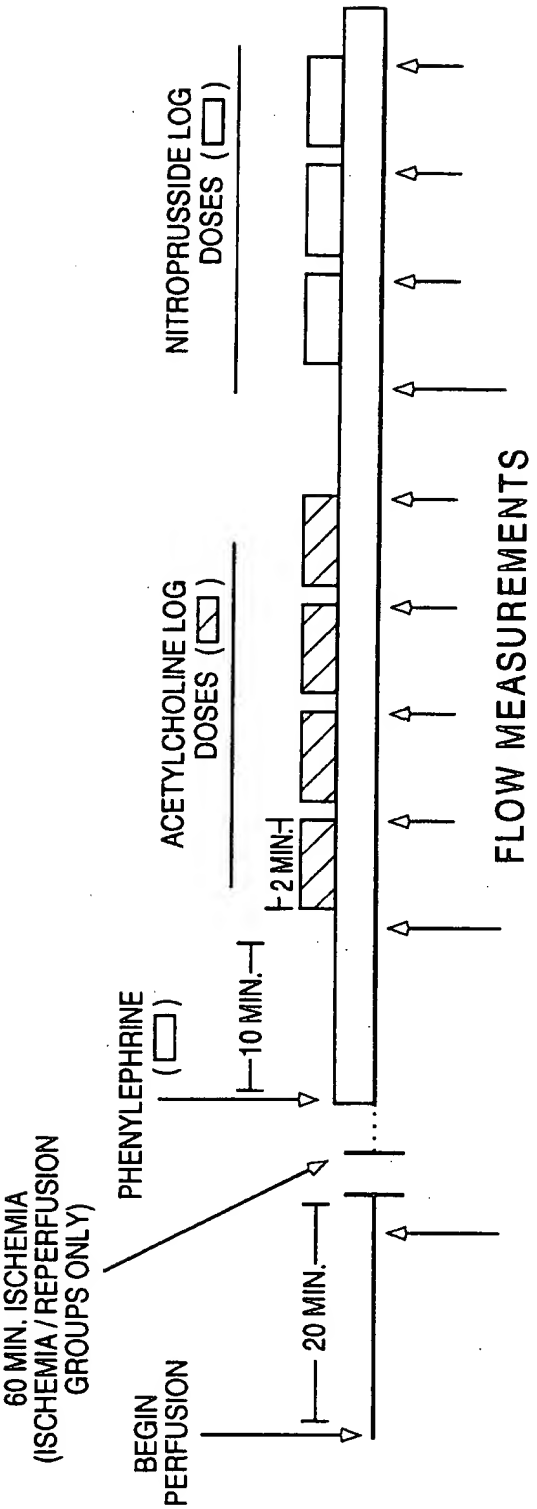
BEGIN PERFUSION
20 MIN.
60 MIN. ISCHEMIA (ISCHEMIA / REPERFUSION GROUPS ONLY)
PHENYLEPHRINE ( )
10 MIN.
2 MIN.
ACETYLCHOLINE LOG DOSES ( )
NITROPRUSSIDE LOG DOSES ( )
FLOW MEASUREMENTS

FIG. 3

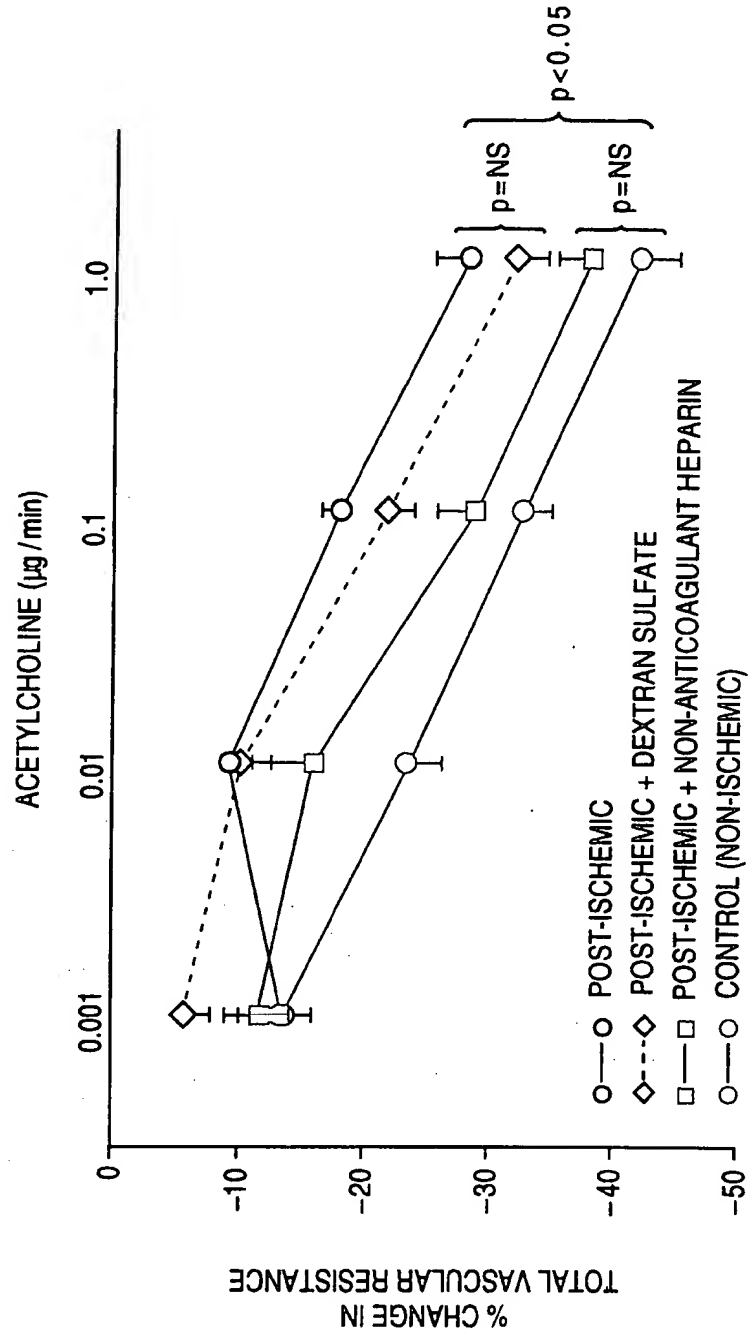
% CHANGE IN
TOTAL VASCULAR RESISTANCE
0
-10
-20
-30
-40
-50
ACETYLCHOLINE (µg/min)
0.001
0.01
0.1
1.0
p<0.05
p=NS
p=NS
POST-ISCHEMIC
POST-ISCHEMIC + DEXTRAN SULFATE
POST-ISCHEMIC + NON-ANTICOAGULANT HEPARIN
CONTROL (NON-ISCHEMIC)

FIG. 4